

## ANTIMICROBIAL PEPTIDES ISOLATED FROM MAST CELLS

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### Related Applications

- This application claims the benefit of United States provisional application  
5 serial number 60/225,354, filed August 15, 2000, the disclosure of which is to be  
incorporated by reference herein in its entirety.

### Government Support

- This invention was made with Government support under University of North  
10 Carolina Sea Grant NA86-RG-0036. The Government has certain rights to this  
invention.

### Field of the Invention

- The present invention relates to antimicrobial peptides exhibiting therapeutic  
15 antimicrobial properties, compositions containing the same, and methods of use  
thereof.

### Background of the Invention

- Infectious diseases are often the most serious impediment to the success of the  
20 commercial aquaculture industry in the United States and worldwide. The epithelial  
surfaces of fish, such as the skin, gills and alimentary tract, provide first contact with  
potential pathogens. It has been reported that several kinds of endogenous  
antimicrobial peptides exist in the skin, stomach and blood of amphibians, mammals  
and insects. Examples include cecropins (insects), defensins (mammals, insects), and  
25 magainins (frogs). These peptides exhibit antimicrobial activity against a broad  
spectrum of organisms. This ability to exhibit broad-spectrum activity provides the  
advantages of nonspecificity and rapid response. These advantages enable the host to  
delay or prevent microbial colonization. Enhancement of nonspecific defenses  
exhibiting a broad range of activity against numerous pathogens may be a cost-

effective method of controlling disease epidemics that inhibit successful aquacultural endeavors.

**Summary of the Invention**

5 A first aspect of the present invention is an antimicrobial compound or endobiotic peptide isolated from fish.

In the course of studying certain endobiotic peptides described herein, it was unexpectedly discovered that these antimicrobial compounds were found in, or isolated from, mast cells. While mast cells are most often associated with allergic  
10 reactions, their precise role in host defense against disease is uncertain, and no peptide antibiotic has previously been isolated therefrom.

Accordingly, a second aspect of the present is an antimicrobial (and particularly an antibacterial) peptide compound (or endobiotic peptide) isolated from mast cells, including but not limited to fish and mammalian mast cells, and including  
15 synthetic analogs thereof. Insofar as the applicants are aware, no polypeptide antibiotic has previously been isolated from mast cells. When isolated from fish mast cells, such compounds are referred to as "piscidins" herein.

Examples of compounds of the foregoing isolated from mast cells include, but are not limited to, those compounds selected from the group consisting of peptides  
20 having an amino acid sequence selected from the group consisting of: **SEQ ID NO: 1** (piscidin 3); **SEQ ID NO: 2** (piscidin 1); **SEQ ID NO: 3** (piscidin 2).

Additional examples of endobiotic peptides of the present invention are given herein as **SEQ ID NO: 4** and **SEQ ID NO: 6**.

A further aspect of the present invention is a method of treating a microbial  
25 infection in a human or animal subject in need thereof, comprising administering to the subject an antimicrobial polypeptide as described above in an amount effective to treat said microbial infection.

A further aspect of the present invention is a use of an antimicrobial polypeptide as described above for the preparation of a medicament for the treatment  
30 of a microbial infection as described above.

A further aspect of the present invention is a pharmaceutical formulation comprising a compound as described above in a pharmaceutically acceptable carrier.

A further aspect of the present invention is an antibody (*e.g.*, a monoclonal antibody) that specifically binds to a compound as described above.

A further aspect of the invention is a method of treating stress (*e.g.*, disease) in fish, comprising administering an endobiotic peptide as described above to a fish in an amount effective to treat or combat stress therein.

A further aspect of the invention is a nucleic acid (*e.g.*, a DNA) that encodes a peptide as described above.

A further aspect of the invention is a method of treating stress in fish, comprising administering a nucleic acid to the fish (*e.g.*, by injecting the nucleic acid into muscle of the fish) in an amount effective to treat or combat stress (*e.g.*, disease) therein.

#### **Detailed Description of Preferred Embodiments**

The present invention will now be described more fully hereinafter with reference to the accompanying figures, in which preferred embodiments of the invention are shown. This invention may, however, be embodied in different forms and should not be construed as limited to the embodiments set forth herein. Rather these embodiments are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the invention to those skilled in the art.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention belongs. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety.

Amino acid sequences disclosed herein are presented in the amino to carboxy direction, from left to right. The amino and carboxy groups are not presented in the sequence. Amino acids are represented herein in by single letter code.

"Amino acid sequence" as used herein, refers to an oligopeptide, peptide, polypeptide, or protein sequence, and fragment thereof, and to naturally occurring or synthetic molecules. Where "amino acid sequence" is recited herein to refer to an amino acid sequence of a naturally occurring protein molecule, amino acid sequence, and like terms, are not meant to limit amino acid sequence to the complete, native amino acid sequence associated with the recited protein molecule.

“Mast cell” as used herein has its ordinary meaning in the art (*see, e.g., Basic and Clinical Immunology*, 145-147 (D. Stites and A. Terr, 7<sup>th</sup> Ed. 1991) and in general refers to immune system cells filled with large granules, which often stain metachromatically, and which degranulate (release their contents extracellularly) with various stimuli. Many mast cells such as mammalian mast cells are characterized by the presence of a high affinity cell surface IgE receptor and histamine-containing cytoplasmic granules. Fish mast cells are also known as eosinophilic granule cells, but fish mast cell degranulation is not known to be mediated by IgE as with mammalian mast cells. Mast cells from which antimicrobial polypeptides of the present invention may be isolated may be of any species (typically a vertebrate species), including but not limited to fish (examples given below), avian (*e.g.*, chicken, turkey, duck, goose, quail, pheasant), amphibians, reptiles, and mammals (*e.g.*, dog, cat, horse, goat, cow, pig, human).

As used herein, the term “antibody” refers to all types of immunoglobulins, including IgG, IgM, IgA, IgD, and IgE. Of these IgM and IgG are particularly preferred. The antibodies may be monoclonal or polyclonal and may be of any species of origin including (for example) mouse, rat, rabbit, horse, or human, or may be chimeric antibodies. *See, e.g., M. Walker et al., Molec. Immunol.* **26**, 403-11 (1989). Antibodies that bind to the peptides of Endobiotic Family 1 and/or Endobiotic Family 2 can be prepared using intact peptides or fragments containing small peptides of interest as the immunizing antigen. The peptide or oligopeptide used to immunize an animal can be derived from the translation of RNA or synthesized chemically and can be conjugated to a carrier protein, if desired. Commonly used carriers that are chemically coupled to peptides include bovine serum albumin, thyroglobulin, and keyhole limpet hemocyanin. The coupled peptide is then used to immunize the animal (*e.g.*, a mouse, a rat, or a rabbit).

The term “antimicrobial”, as used herein, refers to the ability to slow, reduce, terminate or inhibit the growth of microorganisms. Microorganisms which may be treated with compounds of the present invention include, but are not limited to, fungi, parasites, bacteria, viruses, etc.

The term “biological sample”, as used herein, is used in its broadest sense. A biological sample may include blood, urine, muscle tissue, skin, gills, viscera,

mucosal swab, cell culture, or an aqueous medium housing the subject (e.g. for a fish subject).

As used herein, the term "endobiotic", refers to a naturally-occurring, host-produced antibiotic. The vast majority of these endogenous antibiotics are low molecular weight peptides or proteins that exhibit antimicrobial activity against a wide range of microorganisms, including bacteria, viruses, fungi, metazoan and protozoan parasites (Robinette et. al., (1998) *Cell. Mol. Life Sci.* **54**, 467-475). Examples of endobiotics include cecropins (Bowman, H. (1995) *Ann. Rev. Immunol.* **13**: 61-92; Steiner et al., (1981) *Nature* **292**: 246-248), defensins (Selsted et al., *J. Biol. Chem.* **258**: 14485-14489; Lehrer et al. *Ann. Rev. Immunol.* **11**: 105-128), and magainins (Zasloff, M. (1987) *Proc. Natl. Acad. Sci. USA* **84**: 5449-5453). Endobiotics reported and characterized in fish include lysozyme (Roberts, R. (1989) *Fish Pathology*, 2<sup>nd</sup> ed., Bailliere Tindall, London), the aminosterol antibiotic squalamine (Moore et al., (1993) *Proc. Natl. Acad. Sci. USA* **90**: 1354 - 1358), and histone-like proteins (Robinette et al., (1998) *Cell. Mol. Life Sci.* **54**, 467-475). Other examples include parasin from catfish (Park et al., *FEBS Letters* **437**, 258-262 (1998), misgurin from loach (Park et al., *FEBS Letters* **411**, 173-178 (1997)), pleurocidin from flounder (Cole et al., *Journal of Biological Chemistry* **272**, 12008-12013 (1997)), and a <3.5 KDa peptide from rainbow trout. (V. Smith et al., *Fish & shellfish Immunology* **10**, 243-260 (2000)).

"Fish", as used herein, refers to any species of fish susceptible to infectious diseases, particularly bony fishes belonging to the class *Osteichthyes*, and more particularly its subclass *Actinopterygii*. Such examples include hybrid striped bass, *Morone saxatilis* x *Morone chrysops*, channel catfish, *Ictalurus punctatus*, members of the Family Salmonidae, including members of the genus *Oncorhynchus* and *Salmo* such as rainbow trout, *Oncorhynchus mykiss*, flounders (Pleuronectidae and related families, carps (Family Cyprinidae), sturgeons (Family Acipenseridae), sunfish (Family Centrarchidae), mullets (Family Mugilidae), milkfish (*Chanos chanos*), yellow perch (Family Percidae), tilapia (Family Cichlidae), etc.

As used herein, the term "peptide" refers to an oligomer of at least two contiguous amino acid residues.

Subjects which may be treated by the methods of the present invention include

both human and animal subjects. Animal subjects may be of any species, typically vertebrate, including but not limited to birds, fish, reptiles, amphibians, mammals (e.g., dogs, cats, horses, sheep, cows, pigs), etc.

The term "pharmaceutically acceptable" as used herein means that the compound or composition is suitable for administration to a subject to achieve the treatments described herein, without unduly deleterious side effects in light of the severity of the disease and necessity of the treatment.

By "nucleic acid" or "oligonucleotide" or grammatical equivalents herein means at least two nucleotides covalently linked together. A nucleic acid of the present invention will generally contain phosphodiester bonds, although in some cases, as outlined below, nucleic acid analogs are included that may have alternate backbones, comprising, for example, phosphoramidate (Beaucage, *et al.*, TETRAHEDRON, **49** (10): 1925 (1993) and references therein; Letsinger, *J. Org. Chem.*, **35**: 3800 (1970); Sprinzl, *et al.*, Eur. J. Biochem., **81**: 579 (1977); Letsinger, *et al.*, Chemica Scripta, **26**: 141 (1986)), phosphorothioate (Mag, *et al.*, Nucleic Acids Res., **19**: 1437 (1991); and U.S. Patent No. 5, 644, 048), phosphorodithioate (Briu, *et al.*, J. Am. Chem. Soc., **111**: 2321 (1989)), O-methylphosphoroamidite linkages (*see* Eckstein, *Oligonucleotides and Analogues: A Practical Approach*, Oxford University Press), and peptide nucleic acid backbones and linkages (*see* Egholm, J. Am. Chem. Soc., **114**: 1895 (1992); Meier, *et al.*, Chem. Int. Ed. Engl., **31**: 1008 (1992); Nielsen, Nature, **365**: 566 (1993); Carlsson, *et al.*, Nature, **380**: 207 (1996), all of which are incorporated by reference)). Other analog nucleic acids include those with positive backbones (Denpcey, *et al.*, Proc. Natl. Acad. Sci. USA, **92**: 6097 (1995)); non-ionic backbones (U.S. Patent Nos. 5,386,023; 5,637,684; 5,602,240; 5,216,141; and 4,469,863; Kiedrowski, *et al.*, Angew. Chem. Intl. Ed. English, **30**: 423 (1991); Letsinger, *et al.*, J. Am. Chem. Soc., **110**: 4470 (1988); Letsinger, *et al.*, NUCLEOSIDE & NUCLEOTIDE **13**: 1597 (1994); Chapters 2 and 3, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research," Ed. Y.S. Sanghui and P. Dan Cook; Mesmaeker, *et al.*, Bioorganic & Medicinal Chem. Lett., **4**: 395 (1994); Jeffs, *et al.*, J. Biomolecular NMR, **34**: 17 (1994); Tetrahedron Lett (CAPITALIZE), **37**: 743 (1996)) and non-ribose backbones, including those described in U.S. Patent Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC

- Symposium Series 580, "Carbohydrate Modifications in Antisense Research," Ed. Y.S. Sanghui and P. Dan Cook. Nucleic acids containing one or more carbocyclic sugars are also included within the definition of nucleic acids (*see* Jenkins, *et al.*, Chem. Soc. Rev., (1995) pp. 169-176). Several nucleic acid analogs are described in
- 5 Rawls, C & E News, June 2, 1997, page 35. These modifications of the ribose-phosphate backbone may be done to facilitate the addition of additional moieties such as labels, or to increase the stability and half-life of such molecules in physiological environments. In addition, mixtures of naturally occurring nucleic acids and analogs can be made. Alternatively, mixtures of different nucleic acids analogs, and mixtures
- 10 of naturally occurring nucleic acids and analogs may be made. The nucleic acids may be single stranded or double stranded, as specified, or contain portions of both double stranded or single stranded sequence. The nucleic acid contains any combination of deoxyribo-and ribo-nucleotides, and any combination of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xanthine, hypoxanthine, isocytosine,
- 15 isoguanine, *etc.*

- "Fish health", as used herein, refers to the physiological and behavioral responses of fish to stress. Stress is a major predisposing factor for infectious disease in fish (Meyer, F (1970) Seasonal Fluctuations in the Incidence of Disease on Fish Farms. In: Snieszko, S (ed) A Symposium on Diseases of Fishes and Shellfishes.
- 20 Special Publication no 5, American Fisheries Society, Washington, DC; Walters, G and Plumb, J (1980) *J. Fish Biol.* 17: 177-185; Barton (1997) Stress in Finfish: Past, Present, and Future—A Historical Perspective. In: Iwana *et al.* (eds) Fish Stress and Health in Aquaculture. *Soc. Exper. Biol. Seminar Series* 62: 1-34, Cambridge University Press, New York). A number of stressors commonly associated with
- 25 aquaculture can adversely impact or impair the fish immune system, including crowding (Klinger *et al.* (1983) *Aquaculture* 30: 263-272), handling (Ellsaesser, D and Clem, L. (1986) *J. Fish Biol.* 28: 511-521), temperature fluctuation (Clem *et al.* (1984) *Dev. Comp. Immunol.* 8: 313-322; Miller, N. and Clem, L. (1984) *J. Immunol.* 133: 2356-2359) and poor water quality (Smart, G (1981) Aspects of Water Quality
- 30 Producing Stress in Intensive Fish Culture. In Pickering, A (ed) Stress and Fish, Academic Press, London, p 277-294; Schwedler *et al.* (1985) Non-infectious Diseases. In: Tucker, C (ed) Channel Catfish Culture. Developments in Aquaculture

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and Fisheries Science. Vol 15, Elsevier, Amsterdam, p 497-541). Fish health also includes the immune response of the fish. Thus, the terms treating or combating stress as used herein include enhancing or improving fish immune function that has been impaired, or will be impaired, by a stressor.

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#### **A. Peptide production and pharmaceutical formulations**

The methods for making peptides entail, unless otherwise noted, conventional techniques of synthetic organic chemistry, protein chemistry, molecular biology, microbiology, and recombinant DNA technology, which are within the skill of one in the art. Such techniques are fully explained in the literature. See, e.g., Scopes (1987), *Protein Purification Principles and Practice*, 2d Ed, Springer-Verlag; *Methods in Enzymology*, Colowick and Kaplan, eds., Academic Press, Inc.; Sambrook *et al.*, (1989) *Molecular Cloning: A Laboratory Manual* (2d Ed. 1989) (Cold Spring Harbor, NY); *Handbook of Experimental Immunology*, (1986) Vols. I-V, Weir and Blackwell, eds, Blackwell Scientific Publications; House (1972), *Modern Synthetic Reactions*, 2d ed., Benjamin/Cummings, Menlo Park, Calif; Arherton and Sheppard (1989), *Solid Phase Peptide Synthesis: A Practical Approach*, Oxford University Press; Steward and Young, *Solid Phase Peptide Synthesis* (1984), 2d Ed., Pierce Chemical Co.

The peptides of the present invention may optionally be extended at the N-terminus, the C-terminus, or both termini, by the addition of 1 to 4, 5 or 10 amino acids, or more. In addition, or for example, dimers or trimers of the peptides of the invention may be formed in accordance with known techniques, such as the linkage of cysteines by a disulfide bridge or bond.

Preferably, peptides of the invention (particularly synthetic variants of the naturally occurring peptides) are at least 10, 11, 14 or 15 amino acids in length. In preferred embodiments the peptides are not greater than 25 or 30 amino acids in length.

The present invention is also intended to encompass analogs of peptides isolated from mast cells, and the use of such compounds (including nucleic acid intermediates thereof) in compositions and methods as described herein. An "analog" is a chemical compound similar in structure to a first compound, and having either a similar or opposite physiologic action as the first compound.



Methods for determining peptide three-dimensional structure and analogs thereto are known, and are sometimes referred to as "rational drug design techniques". See, e.g., U.S. Patent No. 4,833,092 to Geysen; U.S. Patent No. 4,859,765 to Nestor; U.S. Patent No. 4,853,871 to Pantoliano; U.S. Patent No. 4,863,857 to Blalock; (applicants specifically intend that the disclosures of all U.S. Patent references cited herein be incorporated by reference herein in their entirety). See also Waldrop, *Science* **247**, 28029 (1990); Rossmann, *Nature* **333**, 392 (1988); Weis et al., *Nature* **333**, 426 (1988); James et al., *Science* **260**, 1937 (1993) (development of benzodiazepine peptidomimetic compounds based on the structure and function of tetrapeptide ligands).

In general, those skilled in the art will appreciate that minor deletions or substitutions may be made to the amino acid sequences of peptides of the present invention without unduly adversely affecting the activity thereof. Thus, peptides containing such deletions or substitutions are a further aspect of the present invention. In peptides containing substitutions or replacements of amino acids, one or more amino acids of a peptide sequence may be replaced by one or more other amino acids wherein such replacement does not affect the function of that sequence. Such changes can be guided by known similarities between amino acids in physical features such as charge density, hydrophobicity/hydrophilicity, size and configuration, so that amino acids are substituted with other amino acids having essentially the same functional properties. For example: Ala may be replaced with Val or Ser; Val may be replaced with Ala, Leu, Met, or Ile, preferably Ala or Leu; Leu may be replaced with Ala, Val or Ile, preferably Val or Ile; Gly may be replaced with Pro or Cys, preferably Pro; Pro may be replaced with Gly, Cys, Ser, or Met, preferably Gly, Cys, or Ser; Cys may be replaced with Gly, Pro, Ser, or Met, preferably Pro or Met; Met may be replaced with Pro or Cys, preferably Cys; His may be replaced with Phe or Gln, preferably Phe; Phe may be replaced with His, Tyr, or Trp, preferably His or Tyr; Tyr may be replaced with His, Phe or Trp, preferably Phe or Trp; Trp may be replaced with Phe or Tyr, preferably Tyr; Asn may be replaced with Gln or Ser, preferably Gln; Gln may be replaced with His, Lys, Glu, Asn, or Ser, preferably Asn or Ser; Ser may be replaced with Gln, Thr, Pro, Cys or Ala; Thr may be replaced with Gln or Ser, preferably Ser; Lys may be replaced with Gln or Arg; Arg may be replaced with Lys, Asp or Glu, preferably Lys or Asp; Asp may be replaced with Lys, Arg, or Glu, preferably Arg or Glu; and Glu may be replaced with Arg or Asp,

preferably Asp. Once made, changes can be routinely screened to determine their effects on function by, for example, testing for activity against microorganisms.

Analogs may also be developed by generating a library of molecules, selecting for those molecules which act as ligands for a specified target, and identifying and  
5 amplifying the selected ligands. See, e.g., Kohl et al., *Science* **260**, 1934 (1993) (synthesis and screening of tetrapeptides for inhibitors of farnesyl protein transferase, to inhibit *ras* oncoprotein dependent cell transformation). Techniques for constructing and screening combinatorial libraries of oligomeric biomolecules to identify those that specifically bind to a given receptor protein are known. Suitable oligomers include  
10 peptides, oligonucleotides, carbohydrates, nonoligonucleotides (e.g., phosphorothioate oligonucleotides; see *Chem. and Engineering News*, page 20, 7 Feb. 1994) and nonpeptide polymers (see, e.g., "peptoids" of Simon et al., *Proc. Natl. Acad. Sci. USA* **89**, 9367 (1992)). See also U.S. Patent No. 5,270,170 to Schatz; Scott and Smith, *Science* **249**, 386-390 (1990); Devlin et al., *Science* **249**, 404-406 (1990); Edgington,  
15 *BIOTechnology* **11**, 285 (1993). Peptide libraries may be synthesized on solid supports, or expressed on the surface of bacteriophage viruses (phage display libraries). Techniques are known in the art for screening synthesized molecules to select those with the desired activity, and for labeling the members of the library so that selected active molecules may be identified. See, e.g., Brenner and Lerner, *Proc. Natl. Acad. Sci. USA*  
20 **89**, 5381 (1992) (use of genetic tag to label molecules in a combinatorial library); PCT US93/06948 to Berger et al., (use of recombinant cell transformed with viral transactivating element to screen for potential antiviral molecules able to inhibit initiation of viral transcription); Simon et al., *Proc. Natl. Acad. Sci. USA* **89**, 9367 (1992) (generation and screening of "peptoids", oligomeric N-substituted glycines, to identify  
25 ligands for biological receptors); U.S. Patent No. 5,283,173 to Fields et al., (use of genetically altered *Saccharomyces cerevisiae* to screen peptides for interactions).

As used herein, "combinatorial library" refers to collections of diverse oligomeric biomolecules of differing sequence, which can be screened simultaneously for activity as a ligand for a particular target. Combinatorial libraries may also be  
30 referred to as "shape libraries", i.e., a population of randomized polymers which are potential ligands. The shape of a molecule refers to those features of a molecule that

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govern its interactions with other molecules, including Van der Waals, hydrophobic, electrostatic and dynamic.

# **B. Pharmaceutical formulations and methods of use.**

5           Pharmaceutical formulations of the present invention comprise compounds of the present invention in a pharmaceutically acceptable carrier. Suitable pharmaceutical formulations include those suitable for inhalation, oral, rectal, topical, (including buccal, sublingual, dermal, vaginal and intraocular), parenteral (including subcutaneous, intradermal, intramuscular, intravenous, intraperitoneal, and  
10           intraarticular) and transdermal administration. The compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art. The most suitable route of administration in any given case may depend upon the anatomic location of the condition being treated in the subject, the nature and severity of the condition being treated, and the particular  
15           pharmacologically active compound which is being used. The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art.

          In the manufacture of a medicament according to the invention (the "formulation"), pharmacologically active compounds or the physiologically  
20           acceptable salts thereof (the "active compounds") are typically admixed with, inter alia, an acceptable carrier. The carrier must, of course, be acceptable in the sense of being compatible with any other ingredients in the formulation and must not be deleterious to the patient. The carrier may be a solid or a liquid, or both, and is preferably formulated with the compound as a unit-dose formulation, for example, a  
25           tablet, which may contain from 0.5% to 99% by weight of the active compound. One or more active compounds may be incorporated in the formulations of the invention, which formulations may be prepared by any of the well known techniques of pharmacy consisting essentially of admixing the components, optionally including one or more accessory therapeutic ingredients.

30           The therapeutically effective dosage of any specific pharmacologically active compound identified by methods on the invention, the use of which compounds is in the scope of the present invention, will vary somewhat from compound to compound,

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and subject to subject, and will depend upon the condition of the patient and the route of delivery.

Peptides of the present invention are active against a variety of gram negative and gram positive bacteria, including some bacteria that are resistant to other types of antibiotics. Examples of bacteria which may be treated by the methods of the present invention include but are not limited to those given in **Tables 1-3** below.

In addition, compounds of the present invention are active in treating other microorganisms, including but not limited to fungi, yeasts, protozoa, parasites, etc.

A further aspect of the present invention relates to prior findings by others that some peptide antibiotics can reverse the resistance of antibiotic-resistant bacteria, allowing them to once again become susceptible to conventional antibiotics which had previously been ineffective. That is, if one exposes a bacterium that is resistant to methicillin to a peptide antibiotic, it once again becomes susceptible to methicillin. Thus, stimulating the body's natural production of peptide antibiotics (using an immunostimulant such as beta-glucan), might allow one to once again effectively treat with the conventional antibiotic. Thus, the present invention provides a method of treating, reducing or combating antibiotic resistance in a bacteria, which bacteria is resistant to at least one antibiotic. The method comprises administering to the bacteria, *in vitro* or *in vivo* in a subject in need thereof, a compound of the present invention in an amount effective to reduce antibiotic resistance (*e.g.*, render the bacteria susceptible to subsequent treatment or control with the antibiotic to which it was previously resistant). Examples of the at least one antibiotic to which the bacteria may be resistant. include methicillin, vancomycin, and streptogramin. Example bacteria which may be antibiotic resistant and may be treated by the method of the invention include but are not limited to *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus faecalis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Shigella flexneri*.

Applications for the novel antimicrobial peptides of the present invention include treating stress or disease, particularly bacterial disease, in fish and monitoring fish health. Various stresses cause a decrease in endobiotic levels before the fish show any signs of disease. (Compare Noga, E.J., D.P. Engel, T.W. Arroll, S. McKenna and M. Davidian. 1994. Low serum antibacterial activity coincides with increased

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prevalence of shell disease in blue crabs, *Callinectes sapidus*. Diseases of Aquatic Organisms 19:121-128).

Thus, measurement of these novel endobiotic peptides may provide an indication of chronic and/or acute stress in fish as well as provide an early indication of potential health problems in fish. The inverse relationship between endobiotic levels and stress also provides the basis for assessment of freshness of a fish food product.

Also, these novel peptides may act as cytokines. There is evidence that the cell type containing the 2500 Da peptide of the present invention is the mast cell. Mast cells are known to attract other types of immune cells during inflammatory events in mammals, and there is also evidence for this mechanism in fish. Therefore, the peptides of the present invention may be involved in this chemoattraction. Currently, a number of cytokines are being examined as human therapeutic agents in various diseases including cancer.

Additionally, the novel endobiotic peptides of the present invention may also possess neuroactive function. It is highly likely that these novel peptides interact with target membranes in their interaction with microbes. This interaction most likely involves channel formation. Note that another peptide antibiotic isolated from flounder has both antibacterial and neurological activity (Oren Z and Y Shai. 1996. A class of highly potent antibacterial peptides derived from pardaxin, a pore-forming peptide from the Moses sole fish *Pardachirus marmoratus*. Eur. J. Biochem. 237:304-310).

### C. Antibodies

Antibodies that specifically bind to the peptides of the present invention (*i.e.*, antibodies which bind to a single antigenic site or epitope on the peptides) are useful for a variety of diagnostic purposes, and for screening for additional compounds of the invention in different tissues or species.

Antibodies to SEQ ID NO: 1, SEQ ID NO: 2, and/or SEQ ID NO 3 may be generated using methods that are well known in the art. Such antibodies may include, but are not limited to, polyclonal, monoclonal, chimeric, single chain, Fab fragments, and fragments produced by a Fab expression library.

For the production of antibodies, various hosts including goats, rabbits, rats,

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mice, humans, and others, may be immunized by injection with the endobiotic peptides or any fragment or oligopeptide thereof which has immunogenic properties. Depending on the host species, various adjuvants may be used to increase immunological response. Such adjuvants and/or carriers include, but are not limited to, Freund's, mineral gels such as aluminum hydroxide, and surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol. Among adjuvants used in humans, BCG (bacilli Calmette-Guerin) and *Corynebacterium parvum* are especially preferable.

It is preferred that the oligopeptides, peptides, or fragments used to induce antibodies to the endobiotic peptides have an amino acid sequence consisting of at least five amino acids and more preferably at least 10 amino acids.

Monoclonal antibodies to the endobiotic peptides may be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique, the human B-cell hybridoma technique, and the EBV-hybridoma technique. See, e.g., Kohler, G. *et al.* (1975) *Nature*, **256**: 495-497; Kozbor *et al.* (1985) *J. Immunol. Methods* **81**: 31-42; Cote *et al.* (1983) *Proc. Natl. Acad. Sci. USA* **80**: 2026-2030; Cole *et al.* (1984) *Mol. Cell Biol.* **62**: 109-120.

Various immunoassays may be used for screening to identify antibodies having the desired specificity. Numerous protocols for competitive binding or immunoradiometric assays using either polyclonal or monoclonal antibodies with established specificities are well known in the art. Such immunoassays typically involve the measurement of complex formation between the endobiotic peptide and its specific antibody. A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering endobiotic peptide epitopes is preferred, but a competitive binding assay may also be employed.

Antibodies may be conjugated to a solid support suitable for a diagnostic assay (e.g., beads, plates, slides or wells formed from materials such as latex or polystyrene) in accordance with known techniques, such as precipitation. Antibodies may likewise be conjugated to detectable groups such as radiolabels (e.g., <sup>35</sup>S, <sup>125</sup>I, <sup>131</sup>I), enzyme labels (e.g., horseradish peroxidase, alkaline phosphatase), and fluorescent labels (e.g., fluorescein) in accordance with known techniques.

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Kits for determining if a sample contains proteins of the present invention will include at least one reagent specific for detecting the presence or absence of the protein. Diagnostic kits for carrying out antibody assays may be produced in a number of ways. In one embodiment, the diagnostic kit comprises (a) an antibody  
5 which binds proteins of the present invention conjugated to a solid support and (b) a second antibody which binds peptides of the present invention conjugated to a detectable group. The reagents may also include ancillary agents such as buffering agents and protein stabilizing agents, *e.g.*, polysaccharides and the like. The diagnostic kit may further include, where necessary, other members of the signal-  
10 producing system of which system the detectable group is a member (*e.g.*, enzyme substrates), agents for reducing background interference in a test, control reagents, apparatus for conducting a test, and the like. A second embodiment of a test kit comprises (a) an antibody as above, and (b) a specific binding partner for the antibody conjugated to a detectable group. Ancillary agents as described above may likewise  
15 be included. The test kit may be packaged in any suitable manner, typically with all elements in a single container along with a sheet of printed out instructions for carrying out the test.

#### **D. Nucleic acid production and administration**

20 This invention also encompasses the nucleic acid molecules that encode the peptides described herein. Methods of nucleic acid production are well known to those skilled in the art, and the nucleic acids of the present invention are formulated essentially in the manner previously described for peptide production. By "nucleic acid" or "oligonucleotide" or grammatical equivalents herein means at least two  
25 nucleotides covalently linked together. A nucleic acid of the present invention will generally contain phosphodiester bonds, although in some cases, as outlined below, nucleic acid analogs are included that may have alternate backbones, comprising, for example, phosphoramidate (Beaucage, *et al.*, Tetrahedron, **49** (10): 1925 (1993) and references therein; Letsinger, *J. Org. Chem.*, **35**: 3800 (1970); Sprinzl, *et al.*, Eur. J. Biochem., **81**: 579 (1977); Letsinger, *et al.*, Chemica Scripta, **26**: 141 (1986)),  
30 phosphorothioate (Mag, *et al.*, Nucleic Acids Res., **19**: 1437 (1991); and U.S. Patent No. 5, 644, 048), phosphorodithioate (Briu, *et al.*, J. Am. Chem. Soc., **111**: 2321

- (1989)), O-methylphosphoramidite linkages (*see* Eckstein, *Oligonucleotides and Analogues: A Practical Approach*, Oxford University Press), and peptide nucleic acid backbones and linkages (*see* Egholm, *J. Am. Chem. Soc.*, **114**: 1895 (1992); Meier, *et al.*, *Chem. Int. Ed. Engl.*, **31**: 1008 (1992); Nielsen, *Nature*, **365**: 566 (1993); Carlsson, *et al.*, *Nature*, **380**: 207 (1996), all of which are incorporated by reference)). Other analog nucleic acids include those with positive backbones (Denpoy, *et al.*, *Proc. Natl. Acad. Sci. USA*, **92**: 6097 (1995)); non-ionic backbones (U.S. Patent Nos. 5,386,023; 5,637,684; 5,602,240; 5,216,141; and 4,469,863; Kiedrowski, *et al.*, *Angew. Chem. Intl. Ed. English*, **30**: 423 (1991); Letsinger, *et al.*, *J. Am. Chem. Soc.*, **110**: 4470 (1988); Letsinger, *et al.*, *Nucleoside & Nucleotide* **13**: 1597 (1994); Chapters 2 and 3, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research," Ed. Y.S. Sanghui and P. Dan Cook; Mesmaeker, *et al.*, *Bioorganic & Medicinal Chem. Lett.*, **4**: 395 (1994); Jeffs, *et al.*, *J. Biomolecular NMR*, **34**: 17 (1994); *Tetrahedron Lett.*, **37**: 743 (1996)) and non-ribose backbones, including those described in U.S. Patent Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research," Ed. Y.S. Sanghui and P. Dan Cook. Nucleic acids containing one or more carbocyclic sugars are also included within the definition of nucleic acids (*see* Jenkins, *et al.*, *Chem. Soc. Rev.*, (1995) pp. 169-176). Several nucleic acid analogs are described in Rawls, *C & E News*, June 2, 1997, page 35. These modifications of the ribose-phosphate backbone may be done to facilitate the addition of additional moieties such as labels, or to increase the stability and half-life of such molecules in physiological environments. In addition, mixtures of naturally occurring nucleic acids and analogs can be made. Alternatively, mixtures of different nucleic acids analogs, and mixtures of naturally occurring nucleic acids and analogs may be made. The nucleic acids may be single stranded or double stranded, as specified, or contain portions of both double stranded or single stranded sequence. The nucleic acid contains any combination of deoxyribo- and ribo-nucleotides, and any combination of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xanthine, hypoxanthine, isocytosine, isoguanine, *etc.*

Administration of the nucleic acids of the present invention may be administered according to the methods disclosed in Felgner *et al.* U.S. Patent No.

09929787-081401



5,580,859, or Wolff *et al.*, U.S. Patent No. 5,693,622 (applicants specifically intend that the disclosures of all U.S. Patent references cited herein be incorporated by reference herein in their entirety). Polynucleotide sequences comprising DNA or RNA molecules that are free from any delivery vehicle that acts to facilitate entry into the cell, can be directly administered by injection into tissues. These naked polynucleotide sequences lead to the expression of the endobiotic peptides of the present invention within the subject thereby exerting a pharmacological effect.

The following Examples are provided to illustrate the present invention, and should not be construed as limiting thereof.

### EXAMPLE 1

#### Isolation of Antimicrobial Peptides from Hybrid Striped Bass

At least two families of antimicrobial peptides which have no known sequence homology to any other polypeptides in the NCBI nr or est databases have been isolated from the gills of hybrid striped bass (*Morone saxatilis* x *Morone chrysops*). Endobiotic Family 1 currently consists of 3 peptides, all 22 amino acids long, with a highly homologous N-terminus stretch. These peptides have a molecular weight of about 2500 Da. Endobiotic Family 2 currently consists of 1 peptide at least 44 amino acids long, with the first 6 of 8 amino acids at the N-terminus homologous to those in Endobiotic Family 1. This peptide currently has a molecular weight of about 5329 Da.

### EXAMPLE 2

#### Determination of Molecular Weight and Amino Acid Sequence

The molecular weight of the antimicrobial peptides purified in Example 1 from Endobiotic Family 1 was determined as 2490 Da, 2570 Da, and 2542 Da by the aid of mass spectroscopy. Further amino acid sequence analysis of these three peptides revealed that they are novel peptides consisting of 22 amino acids represented as:

FIHHIFRGIVHAGRSIGRFLTG [SEQ ID NO: 1] (piscidin 3; P3);

FFHHIFRGIVHVGKTIHRLVTG [SEQ ID NO: 2] (piscidin 1; P1); and

FFHHIFRGIVHVGKTIHKLVTG [SEQ ID NO: 3] (piscidin 2; P2).

The molecular weight of the antimicrobial peptide purified in Example 1 from Endobiotic Family 2 was determined as 5329 Da by the aid of mass spectroscopy. Further amino acid sequence analysis of this peptide revealed that it is a novel peptide consisting of an amino acid sequence represented as:

FFRHLFRGAKAIFRGARQGXRHAHKVVSRYRNRDVPETDNNQEEP

[SEQ ID NO: 4], where X is tryptophan or beta-hydroxytryptophan. It is tentatively believed that beta-hydroxytryptophan is present as X in the natural sequence. Tryptophan as X in a synthetic sequence has potent antibacterial activity, suggesting the tryptophan to beta-hydroxytryptophan modification may not be necessary in antimicrobial activity.

### EXAMPLE 3

#### Measurement of Antimicrobial Activity of the Peptides

The antimicrobial activity of the peptides in Endobiotic Family 1 was measured by assessing its antibacterial activity against *Escherichia coli* (*E. coli*). The potency of these peptides against *E. coli* is comparable to that exhibited by some of the strongest naturally-occurring antibacterial peptides (e.g., maganins).

### EXAMPLE 4

#### Production of Antibodies to the Peptides

A peptide antibody against Endobiotic Family 1 was produced. The peptide HIFR [SEQ ID NO:5] (also corresponding to amino acid positions 1 to 11 of SEQ ID NO: 2 and SEQ ID NO: 3) was chemically conjugated to KLH as a carrier. The preparation was injected into rabbits. Serum from the rabbits was processed over an affinity column having the peptide fragment linked to the to capture antibodies specific for the peptide.

### EXAMPLE 5

#### Histone-like Antimicrobial Protein in Channel Catfish

A partial N-terminal amino acid sequence of a predominate antimicrobial protein found in the skin of channel catfish (*Ictalurus punctatus*) exhibits

09929787-081401

approximately 89% homology with rainbow trout (*Salmo trutta*) histone H2B, and thus was designated histone-like protein (HLP) (Robinette et. al., (1998) *Cell. Mol. Life Sci.* **54**, 467-475). HLPs are broad-spectrum antimicrobial polypeptides that appear to be an important component of nonspecific immunity in the skin of channel catfish (Robinette et. al., (1998) *Cell. Mol. Life Sci.* **54**, 467-475).

Healthy, unstressed fish exhibit consistently high levels of a predominate HLP (HLP-1) when measured using ELISA. Fish exposed to chronic stress consisting of overcrowding and elevated ammonia for 1 week showed significantly depressed levels of HLP-1, and fish exposed to stress for 3 or 4 weeks exhibited further depressed levels of HLP-1 (Robinette, D.W. and Noga, E.J.) The time-dependent decrease in HLP-1 levels was not accompanied by any gross signs of disease (Robinette, D.W. and Noga, E.J.). The suppression of HLP-1 in the absence of clinical signs of disease along with evidence that HLP-1 levels are not affected by acute stresses of capture or sampling, suggests that HLP levels may be a promising indicator for monitoring fish health.

## EXAMPLE 6

### Summation of Example 1-5 Results

Two lines of evidence provided herein demonstrate that Endobiotic Family 1 and Endobiotic Family 2 represent a novel family of antimicrobial peptides. First, thus far, these families have no known sequence homology to any other polypeptides in the NCBI nr or est databases. Second, the peptides of Endobiotic Family 1 exhibit potency against *E. coli* that is comparable to that exhibited by some of the strongest naturally occurring antibacterial peptides.

## EXAMPLE 7

### Isolation and Identification of Antimicrobial Peptides from Mast Cells

The purification and identification of antimicrobial peptides such as piscidins from mast cells can be carried out as described in Robinette, D. et al. Antimicrobial activity in the skin of the channel catfish *Ictalurus punctatus*: characterization of broad-spectrum histone-like antimicrobial proteins. *Cell Mol. Life Sci.* **54**, 467-475

09929787-081401

(1998), except using tissues that contain mast cells, rather than skin, as a source or starting material, and with the addition of running a Mini-Prep™ cell before HPLC.

Vertebrates or invertebrates to be used for isolation of antibiotics should be healthy, actively growing (well-fed) and in their optimum environment (temperature, salinity, etc.). Tissues that contain mast cells as defined herein are known and can be collected from subjects in accordance with standard techniques.

After rapid euthanization by chemical or mechanical means, tissues should be rapidly collected from target organs and immediately boiled in acetic acid. This procedures rapidly inactivates any proteases and other degradative chemicals which may cause loss of activity. Although a purified cell or subcellular preparation can also be used, using the entire tissue has the advantage of apparently protecting many peptides against degradation, possibly because of the large amount of contaminating proteins. Most of these contaminating proteins are then removed with subsequent purification steps.

During all subsequent steps, the presence of antibiotics is detected quantitatively using the radial diffusion assay (RDA) and semi-quantitatively using acid-urea gel electrophoresis (bug blot).

Tissue extract is loaded onto a weak cation exchange column (*e.g.*, CM52, Whatman). The extract is then run slowly on the column, and should then be incubated overnight on the column. Overnight incubation, which is unusual with this type of column, appears to greatly improve the recovery of antibiotics of the present invention.

Column fractions are then lyophilized and tested using RDA and bug blot. Usually all active fractions are pooled together. In some cases, separate peaks of activity are pooled.

Pooled samples are then lyophilized and run on a Bio-Rad Mini-Prep Cell. This separation step is extremely important for effective purification of antimicrobial polypeptides because it appears to eliminate factors which tend to co-elute with these bioactive molecules and thus interfere with purification.

Active fractions are then pooled, lyophilized and then run on a C4 or C18 reverse-phase HPLC column. Samples from individual peaks are then pooled and

09929787-081401

lyophilized. This final purification step usually results in peptides which are more than 90% pure.

### EXAMPLE 8

5

#### Additional Measures of Antimicrobial Activity

The antimicrobial activity of synthetic piscidin 1, piscidin 2 and piscidin 3 (P1, P2 and P3) was tested against a variety of different bacteria. Results are given in Tables 1-3 below.

10

**TABLE 1: ANTIBACTERIAL ACTIVITY OF PISCIDINS**  
**Human Bacteria (used MHB)**

Test #	Species	Antibiotic	MIC	MBC
22	<i>Staphylococcus aureus</i>	P1	3.13 µg/ml	3.13 µg/ml
44	<i>Staphylococcus aureus</i>	P1	3.13 µg/ml	3.13 µg/ml
23	<i>Staphylococcus aureus</i>	P3	3.13 µg/ml	3.13 µg/ml
45	<i>Staphylococcus aureus</i>	P3	3.13 µg/ml	3.13 µg/ml
56	<i>Streptococcus faecalis</i>	P1	3.13 µg/ml	3.13 µg/ml
66	<i>Streptococcus faecalis</i>	P1	3.13 µg/ml	3.13 µg/ml
57	<i>Streptococcus faecalis</i>	P3	25 µg/ml	25 µg/ml
67	<i>Streptococcus faecalis</i>	P3	12.5 µg/ml	12.5 µg/ml
46	<i>Escherichia coli</i>	P1	3.13 µg/ml	3.13 µg/ml
47	<i>Escherichia coli</i>	P3	3.13 µg/ml	3.13 µg/ml
34	<i>Escherichia coli</i> D31	P1	1.57 µg/ml	1.57 µg/ml
48	<i>Escherichia coli</i> D31	P1	1.57 µg/ml	3.13 µg/ml
35	<i>Escherichia coli</i> D31	P3	1.57 µg/ml	3.13 µg/ml
49	<i>Escherichia coli</i> D31	P3	1.57 µg/ml	3.13 µg/ml
60	<i>Escherichia coli</i> D22	P1	3.13 µg/ml	3.13 µg/ml
61	<i>Escherichia coli</i> D22	P3	6.25 µg/ml	12.5 µg/ml
68	<i>Klebsiella pneumoniae</i>	P1	3.13 µg/ml	3.13 µg/ml
69	<i>Klebsiella pneumoniae</i>	P3	6.25 µg/ml	6.25 µg/ml
30	<i>Pseudomonas aeruginosa</i>	P1	12.5 µg/ml	12.5 µg/ml
42	<i>Pseudomonas aeruginosa</i>	P1	12.5 µg/ml	12.5 µg/ml
31	<i>Pseudomonas aeruginosa</i>	P3	25 µg/ml	25 µg/ml
43	<i>Pseudomonas aeruginosa</i>	P3	25 µg/ml	25 µg/ml
26	<i>Shigella flexneri</i>	P1	3.13-6.25	6.25 µg/ml

09929787.081401

Test #	Species	Antibiotic	MIC µg/ml	MBC
54	<i>Shigella flexneri</i>	P1	3.13 µg/ml	3.13 µg/ml
64	<i>Shigella flexneri</i>	P1	3.13 µg/ml	3.13 µg/ml
65	<i>Shigella flexneri</i>	P3	6.25 µg/ml	6.25 µg/ml
27	<i>Shigella flexneri</i>	P3	3.13-6.25 µg/ml	6.25 µg/ml

5 **TABLE 2: ANTIBACTERIAL ACTIVITY OF PISCIDINS**  
**Human Bacteria (used MHB)**

Test #	Species	Case #	Anti- biotic	MIC	MBC
86	<i>Enterococcus faecium</i>	00-1434	P1	3.13 µg/ml	6.25 µg/ml
87	<i>Enterococcus faecium</i>	00-1434	P3	6.25 µg/ml	*
89	<i>Enterococcus faecium</i>	00-1435	P1	3.13 µg/ml	3.13 µg/ml
90	<i>Enterococcus faecium</i>	00-1435	P3	3.13 µg/ml	3.13 µg/ml
92	<i>Enterococcus faecium</i>	00-1436	P1	3.13 µg/ml	3.13 µg/ml
93	<i>Enterococcus faecium</i>	00-1436	P3	3.13 µg/ml	3.13 µg/ml
98	<i>Enterococcus faecium</i>	00-1437	P1	3.13 µg/ml	3.13 µg/ml
99	<i>Enterococcus faecium</i>	00-1437	P3	3.13-6.25 µg/ml	*
101	<i>Enterococcus faecium</i>	00-1438	P1	1.57 µg/ml	1.57 µg/ml
102	<i>Enterococcus faecium</i>	00-1438	P3	3.13 µg/ml	3.13 µg/ml
104	<i>Enterococcus faecalis</i>	00-1439	P1	12.5 µg/ml	12.5 µg/ml
105	<i>Enterococcus faecalis</i>	00-1439	P3	50 µg/ml	50 µg/ml
107	<i>Enterococcus gallinarum</i>	00-1440	P1	12.5 µg/ml	12.5 µg/ml
108	<i>Enterococcus gallinarum</i>	00-1440	P3	50 µg/ml	50 µg/ml
95	<i>Staphylococcus epidermidis</i>	00-1441	P1	3.13 µg/ml	3.13 µg/ml
96	<i>Staphylococcus epidermidis</i>	00-1441	P3	3.13 µg/ml	3.13 µg/ml

**KEYS:**

- P1 = Synthetic peptide NSIE #US98-3A5N (fx 5) - peptide #1 (amidated C), 4,000 µg/ml in 0.01%  
Hac
- P3 = Synthetic peptide NSIE #US98-3A5N (fx 4) - (amidated C), 4,000 µg/ml in 0.01% Hac
- \* = found more than 10 colonies of bacteria in the MHA plates from 3 wells of the lowest concentration having no visible growth of bacteria. The results indicate that P6 may be bacteriostatic to these bacteria.
- 00-1434: Resistance phenotype = vancomycin resistant, Gene = *vanA*  
00-1435: Resistance phenotype = vancomycin resistant, Gene = *vanB*  
00-1436: Resistance phenotype = vancomycin resistant, Gene = *vanA*

0929787-081401  
T04180-2872260

00-1437: Resistance phenotype = vancomycin resistant, Gene = *vanA*  
 00-1436, 00-1439, 00-1440: Resistance phenotype = streptogramin-resistant  
 00-1441: Resistance phenotype = methicillin-resistant

5

**TABLE 3: ANTIBACTERIAL ACTIVITY OF PISCIDINS**  
**Fish Bacteria (used MHB)**

Test #	Species	Antibiotic	MIC	MBC
50	<i>Streptococcus iniae</i>	P1	3.13 µg/ml	6.25 µg/ml
62	<i>Streptococcus iniae</i>	P1	3.13 µg/ml	3.13 µg/ml
51	<i>Streptococcus iniae</i>	P3	3.13 µg/ml	3.13 µg/ml
63	<i>Streptococcus iniae</i>	P3	3.13 µg/ml	3.13 µg/ml
70	<i>Lactococcus garviae</i> <sup>a</sup>	P1	3.13 µg/ml	3.13 µg/ml
71	<i>Lactococcus garviae</i> <sup>a</sup>	P3	6.25 µg/ml	12.5 µg/ml
74	<i>Lactococcus garviae</i> <sup>b</sup>	P1	3.13 µg/ml	3.13 µg/ml
75	<i>Lactococcus garviae</i> <sup>b</sup>	P3	3.13 µg/ml	6.25 µg/ml
76	<i>Lactococcus garviae</i> <sup>b</sup>	P1	3.13 µg/ml	6.25 µg/ml
77	<i>Lactococcus garviae</i> <sup>b</sup>	P3	3.13 µg/ml	6.25 µg/ml
78	<i>Lactococcus garviae</i> <sup>b</sup>	P1	3.13 µg/ml	3.13 µg/ml
79	<i>Lactococcus garviae</i> <sup>b</sup>	P3	3.13 µg/ml	6.25 µg/ml
80	<i>Lactococcus garviae</i> <sup>b</sup>	P1	3.13 µg/ml	3.13 µg/ml
81	<i>Lactococcus garviae</i> <sup>b</sup>	P3	3.13 µg/ml	6.25 µg/ml
72	<i>Lactococcus garviae</i> <sup>c</sup>	P1	3.13 µg/ml	3.13 µg/ml
73	<i>Lactococcus garviae</i> <sup>c</sup>	P3	6.25 µg/ml	6.25 µg/ml
116	<i>Vibrio alginolyticus</i>	P1	3.13 µg/ml	3.13 µg/ml
118	<i>Vibrio alginolyticus</i>	P3	6.25 µg/ml	6.25 µg/ml
117	<i>Aeromonas hydrophila</i>	P1	0.79-1.57 µg/ml	0.79-1.57 µg/ml
119	<i>Aeromonas hydrophila</i>	P3	1.57-3.13 µg/ml	1.57-3.13 µg/ml

## KEYS:

- 10 P1 = Synthetic peptide NSIE #US98-3A5N (fx 5) - peptide #1 (amidated C), 4,000 µg/ml in 0.01% HAC  
 P2 = Synthetic peptide NSIE #US98-3A5N (fx 5) - peptide #2 (amidated C), 4,000 µg/ml in 0.01% HAC  
 P3 = Synthetic peptide NSIE #US98-3A5N (fx 4) - (amidated C), 4,000 µg/ml in 0.01% HAC  
 15 a = resistant to oxytetracycline, kanamycin, benzylpenicillin, florfenicol, erythromycin, enrofloxacin & novobiocin  
 b = resistant to oxutetracycline, erythromycin, lincomycin, doxytetracycline  
 c = has not been tested for antibiotic resistance before

09929787.081401

### EXAMPLE 9

#### Dimeric Peptide

- 5 A dimer of a peptide of the present invention was synthesized as an example analog of peptides of the present invention. The dimer had the sequence: HVIGRFIHHFFCCFFHHIFRGIVH (SEQ ID NO: 6), where CC is linked via a disulfide bridge. This dimer was active against several human pathogens, and had no detectable hemolytic activity.

### EXAMPLE 10

#### Antibiotic Activity in Mouse Mast Cells

- 10 This example illustrates the identification of antibiotic activity in mouse mast cells.
- 15 At least 4 distinct zones of antibacterial activity were found in native gels of extracts from the mouse mast cell line TIB 64. Antibacterial activity was detected by running acidified extracts of cell-cultured TIB 64 cells under native acid-urea gel electrophoresis and then "blotting" the gel onto a lawn of the bacterium *Escherichia coli*. Four distinct zones of clearing were detected in some extracts. Crude TIB 64 extracts also appear to have strong activity against *E. coli*, as detected in a radial
- 20 diffusion assay.
- The foregoing examples are illustrative of the present invention, and are not to be construed as limiting thereof. The invention is described by the following claims, with equivalents of the claims to be included therein.